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SYNTHESIS OF PROSTAGLANDINS AND EICOSANOIDS BY THE MAST CELL SECRETORY GRANULE

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The identification of a non-bilayer phospholipid storage in the secretory granule and the linking of the eicosanoid production with the release of histamine have prompted us to examine whether the secretory granule may also serve as both the source as well as the site of prostaglandin synthesis during exocytosis. By exposing the contents of purified granules to exogenous arachidonic acid at neutral pH, we observed the rapid formation of many eicosanoids. The presence of prostaglandins E_2, D_2 and F_{2a} were identified. The kinetics of E_2 formation was also followed. The localization of the arachidonic acid cascade to the secretory granule explains why the production of eicosanoids is so intimately tied to the process of granule exocytosis.

Eicosanoid production and phospholipid turnover have been linked to mast cell granule exocytosis (1,2). The products of the arachidonic acid cascade, such as prostaglandins, leukotrienes, and thromboxanes, are main constituents of the so-called "slow reacting substance of anaphylaxis" (SRS-A) (1,3). Like many other cell types, the mast cell can incorporate exogenous arachidonic acid into its cellular phospholipid. The stimulation of mast cells which have been pre-loaded with radioactive arachidonic acid can result in the release of both labeled arachidonic acid and eicosanoids (4,5). However, the phospholipid pool which provides the arachidonic acid for the synthesis of eicosanoids has not been identified.

Recently, we found that the secretory granule of the quiescent mast cell contains a large amount of matrix-bound phospholipid which exists in non-bilayer form[a]. This pool of phospholipid is believed to be responsible for sustaining the process of de novo membrane assembly which takes place during secretory granule activation (6-9). On activation of the granule, some of this phospholipid rapidly assembles into bilayer vesicles which insert into

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Abbreviations used: PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; PGF_{2a}, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2; LTB_4, leukotriene B_4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; RIA, radioimmunoassay; TLC, thin layer chromatography.

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the perigranular membrane. The insertion of these newly assembled vesicles enables the perigranular membrane of the activated granule to enlarge and approach contact with other membranes. The contact between the enlarging perigranular membrane and the plasma membrane results in their fusion and the formation of a pore through which the granule contents are extruded. Since this membrane-expanding event may not require the complete utilization of the matrix-stored phospholipid, a significant amount of it may be secreted with other granule components during exocytosis. This remnant phospholipid which also contains arachidonic acid, can serve as the substrate needed for the synthesis of eicosanoids (10). If the enzymes of the arachidonic acid cascade are also present in the granule matrix along with the phospholipid, a concomitant activation of these enzymes during granule activation would account for the parallel kinetics of prostaglandin production with the release of histamine during anaphylaxis.

In this communication, we present evidence which suggests the presence of the enzymes of the arachidonic acid cascade within the secretory granule of the mast cell. By simply exposing [1-^{14}C]-arachidonic acid to the contents of purified granules at neutral pH, we have detected the rapid formation of at least 10 different arachidonic acid metabolites. From these, the presence of PGE_2, PGD_2, and PGF_{2a} have been identified. Based on these results, we conclude that the secretory granule can serve as the source as well as the site of prostaglandin and eicosanoid synthesis during histamine release. This also implies that the process of granule activation is accompanied by the initiation of the arachidonic acid cascade leading to the production of the various lipid-derived mediators during exocytosis.

MATERIALS AND METHODS The procedure for granule preparation is similar to that which has been described for the determination of granule phospholipid contents[a]. Briefly, purified mast cells were obtained from Sprague-Dawley rats according to published procedure using a serum albumin gradient. Granules were obtained by graded sonication of the mast cells using a sonifier (Bramson W350) equipped with a microprobe. The combined supernatants which contained the granules were centrifuged at 72xg for 10 min to remove aggregates. The granules were pelleted at 960xg for 15 min. To avoid cytosolic contamination, granules were washed by resuspension in buffer and pelleted at 960xg. The purity of the granule can be seen in Fig. 1. Routine electron microscopy was carried out as described previously (6-7).

To assay for prostaglandins, the granule pellet was resuspended in an assay buffer containing 0.1% (w/v) digitonin (Fluka Bio Chemika), 0.5 mM $CaCl_2$, 0.05 mg/ml PMSF, 0.1 mg/ml leupeptin (Sigma Chemicals), 10 mM HEPES pH 6.8, and 37 uM of [1-^{14}C]-arachidonic acid (New England Nuclear, Du Pont). After a brief sonication (3x 3-sec burst), the reaction mixture was incubated in room temperature (21 ^{o}C) for a specified length of time. The reaction was quenched with cold acetone and prostaglandins were extracted according to the procedure of Salmon and Flower (11). The solvent systems for TLC were: (I) the organic phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (110:50:20:100, v/v/v/v) and (II) diethylether:methanol:acetic acid (90:1:2, v/v/v) (11,12). PGE_2 was also verified and quantitated using an RIA procedure (New England Nuclear, Du Pont). The HP-K high performance TLC plates were

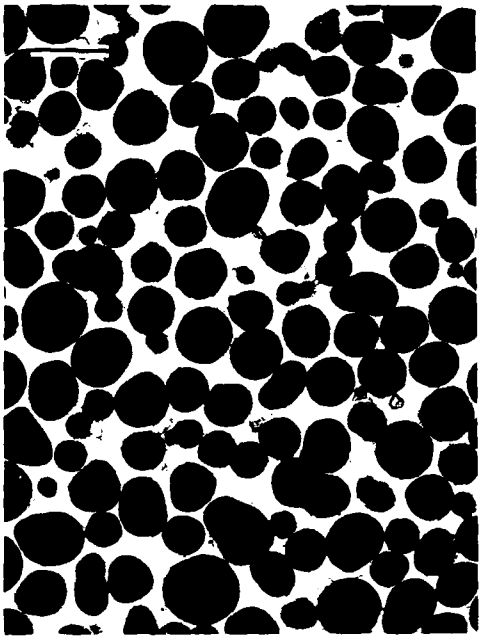

Fig. 1. Electron micrograph of the purified mast cell granules. The bar in the lower right corner represents 1 um.

from Whatmann and were used without pre-activation. Eicosanoid standards were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals used were of reagent grade.

RESULTS By exposing a small amount of [1-^{14}C]-arachidonic acid to the contents of purified granules at 21 ^{o}C, pH 6.8, we can demonstrate a rapid conversion of this labeled substrate into various eicosanoids including PGE_2 and PGD_2. A typical result of such an experiment is shown in Fig. 2. In this figure, lane A is the control while lanes B, C, and D represent individual samples incubated for 0, 10, and 30 min respectively. The main reaction products are distributed in 7 major bands with the approximate Rf values of 0, 0.23, 0.37, 0.55, 0.64, 0.75, and 0.91 respectively. When compared with the Rf values of standard eicosanoids, Band 1, Band 2, Band 3, Band 4, Band 5, and Band 7 have similar mobility as phospholipid, PGF_{2a}, PGE_2, PGD_2, LTB_4, and arachidonic acid, which have corresponding Rf values of 0, 0.24, 0.38, 0.5, 0.61, and 0.95 respectively in the same TLC solvent system.

Fig. 3 shows that when a 5-minute reaction mixture is analyzed using a two-dimensional chromatography system, each of the major bands seen in Fig. 2 can be further resolved into more than one radioactive product. Band 1 has been identified as phospholipid. Band 2 is tentatively identified as PGF_{2a}. Band 3 is made up of at least 2 components and the major one is identified as PGE_2. There is also a spot between band 3 and band 4. Band 4 has relative

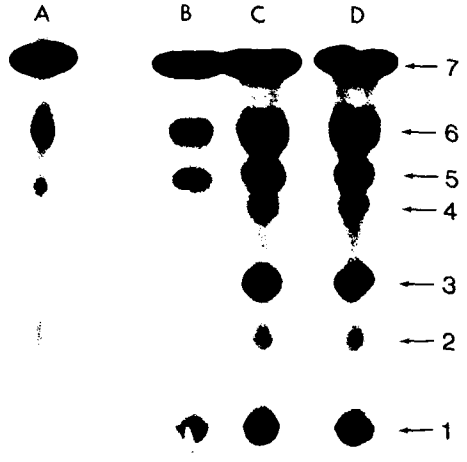

Fig. 2. Autoradiogram of eicosanoids produced by the incubation of [1-^{14}C]-arachidonic acid with dispersed mast cell secretory granules in vitro. Lanes A-D represent chromatograms of control (substrate in reaction mixture without protein), 0 min, 10 min, and 30 min samples incubated at 21°C respectively. The minor spots in the control sample (lane A) are inherent impurities of the substrate. The resulted eicosanoids are resolved into 7 major bands by TLC solvent system (I). The major components of each band are: phospholipids (Band 1), PGF_{2a} (Band 2), PGE_2 (Band 3), PGD_2 (Band 4), and arachidonic acid (Band 7). (Band 5) and (Band 6) contain several unidentified eicosanoids along with the contaminants of arachidonic acid. The Rf values for the various bands are: 0, 0.23, 0.37, 0.55, 0.64, 0.75, and 0.91 respectively.

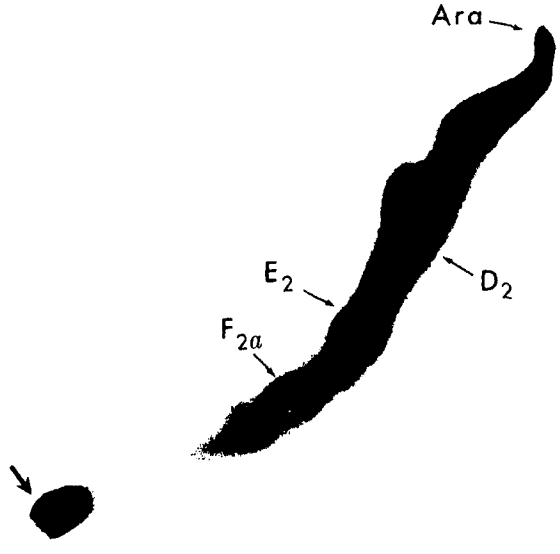

Fig. 3. Two-dimensional TLC resolution of labeled eicosanoids resulted from a 5-minute incubation of [1-^{14}C]-arachidonic acid with granule matrix contents. Sample was spotted at the lower left corner (arrow) and chromatographed in solvent system (I) to resolve the sample vertically into a similar 7-band pattern with Rf values corresponding to those shown in Fig. 2. The horizontal direction was chromatographed in solvent system (II) to further resolve each major band horizontally into several components. Spots for arachidonic acid, PGD_2, PGE_2, and PGF_{2a} have been assigned.

mobility similar to that of PGD_2 which is a known mast cell eicosanoid (1). Band 5 and Band 6 are each composed of at least two different components. One of the spots of Band 5 might be LTB_4 which has similar mobility in this TLC system. The bulk of band 7 is unreacted arachidonic acid. Without counting the components of band 1, there are at least 10 eicosanoids synthesized within 5 min at 21 ^{o}C by the mast cell granule enzymes when exogenous arachidonic acid is added.

When the time course of PGE_2 production is followed by using the RIA procedure, its rate of production appeared quite linear for the first five minutes (Fig. 4). The initial rate of PGE_2 production for this particular experiment is equal to approximately 30 pg/ul/min which corresponds to about 25 pg/min/ug granule protein. The protein concentration is estimated by assuming that an average granule contained about 0.38 pg protein[a] and the assay solution contained approximately $3x10^6$ granules/ul. This rate is expected to vary according to experimental conditions. Our results may not represent those that can be achieved under the optimal assay conditions.

DISCUSSION As much as 18% of the total phospholipid fatty acid of the mast cell is made up of arachidonic acid (13). Since the current dogma assumes that cellular phospholipid exists only in bilayer form, it follows that the phospholipid which provides the arachidonic acid for the eicosanoid synthesis

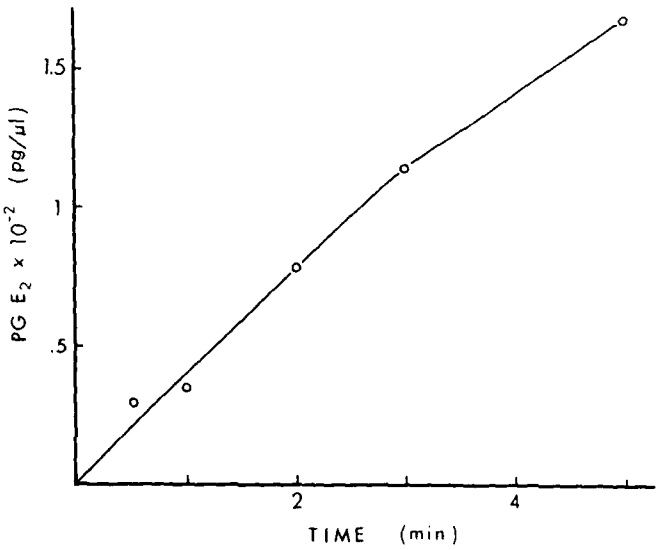

Fig. 4. The time course of prostaglandin E_2 production by granule enzymes as determined by the RIA procedure. The experiment was carried out as described in Materials and Methods. The protein concentration was about 1.2 mg/ml and the added arachidonic acid was 37 µM. The initial rate of about 30 pg/ul/min is equivalent to approximate 25 pg PGE_2/µg protein/min when the granule protein concentration is taken into account.

must also originate from the cellular membrane (14, 15). However, this assumption cannot explain how the arachidonic acid or the phospholipid can become so readily available to the eicosanoid synthesizing enzymes during the stimulation-secretion coupling process. For the mast cell, the antigen challenge of the IgE receptor-mediated exocytosis also results in triggering the arachidonic acid cascade leading to the rapid formation of eicosanoids (1, 16). The initial time course for the eicosanoid production also closely parallels the time course for the histamine release during an anaphylactic reaction (4,16). The time course of histamine release can be very rapid (17). Since receptor activation is a membrane-mediated event, to explain the rapid and intimate coupling of these two processes, it is tempting to assume that the eicosanoid synthesizing enzymes might be localized on the plasma membrane near the IgE receptors. However, such an assumption would also require the availability of a high calcium pool needed for the enzyme activity. To date, the presence of a high calcium pool in association with the plasma membrane has not been demonstrated. The use of immunocytochemical technique has also failed to demonstrate cyclo-oxygenase activity at the plasma membrane (18). Furthermore, the major enzymes for the conversion of PGH_2 to PGD_2 in the eicosanoid biosynthetic pathway are found in the cytosol instead of the microsomal fraction (19). All these contradictions suggest the existence of a different explanation for the intimate linking between the arachidonic acid cascade and the release of histamine.

Our recent identification of a non-bilayer phospholipid storage in the secretory granules of the mast cell raises a strong possibility that this phospholipid pool may be responsible for providing the bulk of the arachidonic acid needed in the synthesis of the eicosanoids during histamine release (10). The presence of a high calcium pool in the granule can easily satisfy the calcium requirement for the various enzyme activities (20). The present finding which localizes the eicosanoid synthesizing enzymes to the secretory granule not only makes the linking of the histamine-release process to the production of eicosanoids a physical possibility, it also explains why the initial time courses for both the histamine release and the production of eicosanoids are so closely parallel. The simultaneous activation of the granule and the initiation of the arachidonic acid cascade provide the basis for the coupling of exocytosis to the formation of lipid-derived mediators.

In this communication, we have demonstrated that exposure of arachidonic acid to the granule matrix contents at neutral pH is sufficient to initiate the arachidonic acid cascade. This finding underscores the important role of pH in granule activation. Many enzymes are inhibited by an acidic pH. In fact the pH of the quiescent granule has been suggested to be about 5 (21). This acidic pH is necessary to suppress the granule protease activity which can cause the elevation of intragranular pH and osmotic pressure. If we are

correct in postulating that an influx of cellular water into the granule is the early event in the activation of the granule, then this water influx may also contribute to local pH changes. If the influx of cellular water were tied to an influx of alkaline ions, this would provide an effective way to raise the intragranular pH and to activate the granule enzymes which include those involved in the arachidonic acid cascade. The possibility for the existence of a K^+/H^+ or Na^+/H^+ exchange mechanism on the perigranular membrane should be investigated.

Following the fusion of the activated granule with the plasma membrane, the granule contents are exteriorized and become exposed to the extracellular pH. This alkalization assures the activation of the various granule enzymes and the continued synthesis of eicosanoids. This continued synthesis may account for the accumulation of PGD_2 detected beyond the time point when the release of histamine has already leveled off or decreased. The exposure of an eicosanoid synthesizing machinery to the extracellular environment may also have profound effects on the production and the breakdown of the mediators. The nonenzymatic conversion of PGH_2 to PGD_2 in the presence of serum albumin is an example of how the local environment can alter the outcome of the arachidonic acid cascade (22).

Although we have not identified any product of the lipoxygenase pathways in this study, yet we have implied that these enzymes, if present, can also be found in the granule along with the cyclo-oxygenase. This implication is based on the fact that many products of the lipoxygenase pathways, such as the leukotrienes, the hydroperoxy- and hydroxy-eicosatetraenoic acids, have already been detected together with prostaglandins and other products of the cyclo-oxygenase pathways during the anaphylactic reaction (1). Although our present experimental conditions may not be conducive for the activity of the lipoxygenases, we suspect that LTB_4 might be present in Band 5 (Fig. 2) since Band 5 contains component of similar Rf value as the LTB_4 standard. Some of the hydroperoxyeicosatetraenoic acids and the hydroxyeicosatetraenoic acids may also be present in Band 6 and Band 7 in Fig. 2. Until proven otherwise, it might be superfluous at this point to postulate a different cellular compartment for the lipoxygenases.

To extrapolate our present findings to the mechanism of anaphylaxis, it is important to realize that the triggering of the arachidonic acid cascade during granule activation also depends on the availability of the substrate, arachidonic acid. This means that the release of the arachidonic acid from the hydrolysis of matrix-bound phospholipid by phospholipases is crucial to the initiation of the arachidonic acid cascade in vivo. In agreement with this, our preliminary data has also suggested the presence of a phospholipase A_2 activity in the mast cell granule, and its presence has enabled us to elucidate the synthesis of PGE_2 from endogenous arachidonic acid. Since the

granules can also rapidly synthesize phospholipids from exogenous arachidonic acid (Band 1 in Fig. 2) it suggests that the granules must also possess the machinery for phospholipid turnover. All these suggest that the secretory granule may hold the key to the many rapid biochemical events associated with exocytosis, which includes de novo membrane assembly, rapid phospholipid turnover, and the production of the lipid-derived mediators.

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REFERENCES

1. Metcalfe, D.D., Kaliner, M., and Donlon, M.A. (1981) CRC Crit. Rev. Immunol. 3, 23-74.
2. Cockcroft, S. (1982) Cell Calcium 3, 337-349.
3. Yecies, L.D., Wedmer, H.J., Johnson, S.M., Jakschik, B.A., and Parker, C.W. (1979) J. Immunol. 122, 2083-2089.
4. Kawabe, H., Hayashi, H., and Hayaishi, O. (1987) Biochem. Biophys. Res. Commun. 143, 467-474.
5. Garcia-Gil, M. and Siraganian, R.P. (1986) J. Immunol. 136, 3825-3828.
6. Chock, S.P. and Schmauder-Chock, E.A (1985) Biochem. Biophys. Res. Commun. 132, 134-139.
7. Schmauder-Chock, E.A. and Chock, S.P. (1987) Histochemical J. 19, 413-418.
8. Chock, S.P. and Chock, E.S. (1985) Fed. Proc. 44, 1324 (5341 abs.).
9. Schmauder-Chock, E.A. and Chock, S.P. (1987) In: Proceedings of the 45th Annual Meeting of the Electron Microscopy Society of America. (Bailey, G.W. ed.) pp.782-783. San Francisco Press.
10. Chock, S.P. and Schmauder-Chock, E. (1987). In: Prostaglandins and Lipid Metabolism in Radiation Injury. (Walden, T.L. and Hughes, H.N., eds.) pp 127-132. Plenum Press, N.Y.
11. Salmon, J.A. and Flower, R.J. (1982) In Methods in Enzymology (Lands, W.E.M. and Smith, W.L. eds.) Vol. 86 pp.477-493. Academic Press N.Y.
12. Bailey, J.M., Bryant, R.W., Feinmark, S.J., and Makheja, A.N. (1977) Prostaglandins 13, 479-492.
13. Strandberg, K. and Westerberg, S. (1976) Mol. Cell. Bioch. 11, 103-107
14. Samuelsson B. (1982) Biosci. Rep. 3, 791-813.
15. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1983) Molecular biology of the cell. p350. Garland Publishing Inc., N.Y. and London.
16. Ishizaka, T. and Ishizaka, K. (1984) Prog. Allergy 34, 188-235.
17. Douglas, W.W. (1974) Biochem. Soc. Symp. 39, 1-28.
18. Rollins, T.E., and Smith, W.L. (1980) J. Biol. Chem. 255, 4872-4875.
19. Shimizu, T., Yamamoto, S., and Hayaishi, O. (1979) J. Biol. Chem. 254, 5222-5228.
20. Chock, E.S., Donlon, M.A., Fiori, C.E., and Catravas, G.N. (1982) J. Cell Biol. 95, 409a.
21. Lagunoff, D. and Rickard, A. (1983) Exptl. Cell Res. 144, 353-360.
22. Hamberg, M. and Fredholm, B.B. (1976) Biochim. Biophys. Acta 431, 189-193.